Kinetics of chronic inflammation in Nile tilapia fed n-3 and n-6 essential fatty acids

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Abstract – The objective of this work was to investigate the effect of dietary supplementation with essential fatty acids on the kinetics of macrophage accumulation and giant cell formation in Nile tilapia (*Oreochromis niloticus*). The supplementation sources were soybean oil (SO, source of omega 6, n-6) and linseed oil (LO, source of omega 3, n-3), in the following proportions: 100% SO; 75% SO + 25% LO; 50% SO + 50% LO; 25% SO + 75% LO; and 100% LO (four replicates per treatment). After a feeding period of three months, growth performance was evaluated, and glass coverslips were implanted into the subcutaneous connective tissue of fish, being removed for examination at 2, 4, 6, and 8 days after implantation. Growth performance did not differ between treatments. Fish fed 100% linseed oil diet had the greatest macrophage accumulation and the fastest Langhans cell formation on the sixth day. On the eighth day, Langhans cells were predominant on the coverslips implanted in the fish feed 75 and 100% linseed oil. n-3 fatty acids may contribute to macrophage recruitment and giant cell formation in fish chronic inflammatory response to foreign body.

Index terms: *Oreochromis niloticus*, foreign body, growth performance, inflammatory response, lipid nutrition.

Cinética da inflamação crônica em tilápia-do-nilo alimentada com ácidos graxos essenciais n-3 e n-6

Resumo – O objetivo deste trabalho foi investigar o efeito da suplementação alimentar com ácidos graxos essenciais sobre a cinética do acúmulo de macrófagos e a formação de células gigantes em tilápia-do-nilo (*Oreochromis niloticus*). As fontes de suplementação foram óleo de soja (OS, fonte de ômega 6, n-6) e óleo de linhaça (OL, fonte de ômega 3, n-3), nas seguintes proporções: 100% OS; 75% OS + 25% OL; 50% OS + 50% OL; 25% OS + 75% OL; e 100% OL (quatro repetições por tratamento). Após período de alimentação de três meses, foi avaliado o desempenho produtivo, e lamínulas de vidro foram implantadas no tecido subcutâneo dos peixes, as quais foram removidas para exame aos 2, 4, 6 e 8 dias após o implante. O desempenho produtivo não diferiu entre os tratamentos. Os peixes alimentados com 100% de óleo de linhaça tiveram maior acúmulo de macrófagos e formação mais rápida de células de Langhans, no sexto dia. No oitavo dia, as células de Langhans foram predominantes nas lamínulas implantadas nos peixes alimentados com 75 e 100% de óleo de linhaça. Os ácidos graxos n-3 podem contribuir para o recrutamento de macrófagos e a formação de células gigantes, na resposta inflamatória crônica a corpo estranho em peixes.

Termos para indexação: *Oreochromis niloticus*, corpo estranho, desempenho produtivo, resposta inflamatória, nutrição lipídica.

Introduction

The participation of fatty acids in nonspecific defense responses is attributed to the production of eicosanoids with pro-inflammatory activity (Balfry & Higgs, 2001). Fish fed diets rich in n-3 and n-6 fatty acids present greater B lymphocyte response and survival after challenge with *Aeromonas salmonicida* and *Vibrio anguillarum* (Thompson et al., 1996). In addition, they have higher antibody titers when immunized against *Edwardsiella ictaluri* (Fracalossi et al., 1994).

Multinucleated giant cells (MGC) have been described among fish with infectious or parasitic diseases (Olsen et al., 2006; Hogge et al., 2008; Jacobs et al., 2009), and in experimental in vitro (Couso et al., 2002) and in vivo (Petric et al., 2003b; Belo et al., 2005) models. Accumulation of inflammatory cells at the lesion site is the main inflammation event to protect the organism against foreign elements (Kumar et al., 2004). MGC formation occurs as an attempt to involve the foreign body in order to isolate it. These macrophage activities are regulated by chemical mediators and

modulators (Kumar et al., 2004). Petric et al. (2003b) demonstrated that the implantation of glass coverslips in the subcutaneous tissue of *Piaractus mesopotamicus* induces the formation of MGC, initially as foreign body giant cells and, later on, as Langhans type.

Food supplementation with vitamin C increases the macrophage activity and induces its accumulation (Petric et al., 2003a). Using the same experimental model, Belo et al. (2005) observed benefic effects of supplementation with vitamin E among teleost fish kept at a high population density. However, there is little information about the influence of essential fatty acids on the activity of macrophages in chronic inflammation of fish.

The objective of this work was to investigate the effect of dietary supplementation with essential fatty acids on the kinetics of macrophage accumulation and giant cell formation in Nile tilapia (*Oreochromis niloticus*).

Materials and Methods

Two hundred Nile tilapia juveniles (69.86±5.01 g) were randomly allocated to 20 glass 310 L fiber tanks (n=10). The tanks were supplied with chlorine-free running water from an artesian well, with a 1 L min^{-1} flow and supplementary aeration.

The fish were weighed and measured at the beginning of the experiment. To do this, they were anesthetized in an aqueous solution of benzocaine (1 g per 10 L). After this initial biometry, they were acclimatized for 15 days. During this phase, a same baseline feed was provided daily for all groups, corresponding to around 3% of the biomass.

After acclimatization, the fish received the experimental diets containing the essential fatty acids for 90 days and had their productive performance evaluated. After this, glass coverslips were implanted into the subcutaneous connective tissue, and the chronic inflammation was evaluated, by quantifying the number of macrophages, polykaryon cells, and giant cell nuclei and types.

Degummed soybean oil (SO) was used as a source of omega 6 (n-6), and linseed oil (LO) as a source of omega 3 (n-3), in the following proportions: 100% SO; 75% SO + 25% LO; 50% SO + 50% LO; 25% SO + 75% LO; and 100% LO. Each treatment consisted of four replicates.

Initially, the baseline diet was standardized such that it contained 28% digestible protein (DP) and 3,200 kcal (13,397.76 kJ) of digestible energy (DE). The composition of the baseline diet followed the nutritional requirements of Nile tilapia (National Research Council, 1993), as shown in Table 1.

After preparing the diets, feed samples were analyzed to determine their essential fatty acid profile. About 4 g of each sample were homogenized for lipid extraction using the method of Folch et al. (1957), followed by esterification of the lipid fraction using the method of Hartman & Lago (1973).

The fatty acid methyl esters were analyzed using a GC-14A Shimadzu gas chromatograph (Shimadzu do Brasil, São Paulo, SP) equipped with a flame ionization detector, a split injector, and Supelco 2560 fused silica capillary columns of 100 m length and 0.25 mm internal diameter. The following operational parameters were used: detector and injector temperatures of 270 and 250°C, respectively; column temperature programmed at 100°C, increasing up to 180°C at 10ºC min^{-1} rate, and from 180 to 240°C at 1°C min^{-1} rate, and holding at this end temperature for 10 min; nitrogen as carrier

Table 1. Composition and chemical-bromatological analysis of the diet.

Ingredient	Content (g kg^{-1})
Soybean bran	330.00
Corn meal	180.00
Wheat bran	150.00
Rice bran	90.00
Fish meal	165.00
Oil[(1)]	60.00
Dicalcium phosphate	10.00
Vitamin and mineral supplement[(2)]	5.00
BHT (antioxidant)	0.20
Calcitic lime	9.80
Composition	
Gross protein (%)	29.15
Gross energy (kJ)	12,861.32
Gross fiber (%)	4.72
Ether extract (%)	10.44
Nitrogen free extract (%)	37.10
Dry matter (%)	90.73

[(1)]The proportions of degummed soybean oil and linseed oil were added according to each treatment. [(2)]Vitamin and mineral supplement composition: vitamin A, 1,200,000 IU; vitamin B1, 4,800 mg; vitamin B12, 4,800 mg; vitamin B2, 4,800 mg; vitamin B6, 4,800 mg; vitamin D3, 200,000 IU; vitamin K3, 2,400 mg; folic acid, 1,200 mg; biotin, 48 mg; calcium pantothenate, 12,000 mg; choline chloride, 108 g; niacin, 24,000 mg; selenium 100 mg; iodine, 100 mg; cobalt, 10 mg; copper, 3,000 mg; iron, 50,000 mg; manganese, 20,000 mg; zinc, 30,000 mg.

gas, at 0.6 mL min^{-1} and linear velocity of 14 cm s^{-1}; split ratio of 1:75 with a total flow of 52 mL min^{-1} and column pressure of 167 kPa. Retention time and peak areas were recorded by microcomputer, using the Class GC 10 software (Shimadzu Corporation, Tokyo, Japan). Dietary fatty acids were expressed in percentage

Water quality was evaluated daily, at feeding times, for temperature (28.17±1.0°C); dissolved oxygen concentration (5.15±0.4 mg L^{-1}); and hydrogen ion potential (7.45±0.06) and electric conductivity (178.5±5.3 mS cm^{-1}), determined using an oximeter YSI-55, (YSI Incorporated, Yellow Springs, OH, USA) and a pH meter YSI-63, (YSI Incorporated, Yellow Springs, OH, USA), respectively. Every fortnight, the ammonia concentration (0.1976±0.095 mg NH_3 L^{-1}) and alkalinity (28.64±1.12 mg $CaCO_3$ L^{-1}) were measured. The results remained within the comfort zone for Nile tilapia (Boyd, 1990).

Daily feed consumption (3% of the biomass), weight gain, food conversion, and specific growth rate were evaluated monthly (30, 60, and 90 days). Food conversion (FC) was estimated with the formula FC = feed consumption/weight gain, and specific growth rate (SGR) with the equation SGR = 100 (ln final weight - ln initial weight/days of experiment).

Ninety days after starting the experiment, ten randomly chosen fish from each treatment were anesthetized and subjected to implantation of a glass coverslip (Petric et al., 2003b). The coverslips were removed after euthanasia 2, 4, 6, and 8 days after the implantation, washed with a 0.65% saline solution, fixed in Bouin's solution, and stained with hematoxylin-eosin. To evaluate the inflammatory response, seven coverslips were chosen randomly from the experimental units. All of the macrophages isolated and the polykaryons formed were counted, as were the number of foreign-body giant cells and the number of Langhans giant cells. The counting was carried out under an optical microscope (400 x), in five fields per coverslip, totalizing 35 fields counted per treatment.

All data were statistically analyzed using a completely randomized design, split plotted in time, with five proportions of fatty acids x four evaluation times. Comparison of the different experimental groups was carried out by applying the analysis of variance procedure (SAS Institute, 2001). Significant differences at 5% probability were estimated with Tukey's test, according to Snedecor & Cochran (1974).

Results and Discussion

With the progressive replacement of soybean oil by linseed oil, the supplementation values for linoleic acid (C18:2n6c) decreased and the linolenic acid values (C18:3n3) increased (Table 2). This result is an evidence that the profile of the fatty acids present in the feed met the initial proposal of the study.

With the evolution of the inflammation, over the course of the experiment, cell accumulation on the glass coverslip increased (Table 3). Two days after the implantation, macrophage number was smaller and few polykaryons with two to five nuclei were formed. On the fourth day, however, the numbers of polykaryons with giant cells containing six to ten nuclei increased. Due to the high rates of cell accumulation on the coverslips, giant cells with more than 20 nuclei and Langhans cells were already present on the sixth and eighth days (Figure 1). There was a remarkable increase in the occurrence of these polykaryon cells between these days (Table 3). However, a reduction in the number of macrophages was observed on the eighth day due to the space occupied by the great number of polykaryon cells attached to the coverslips.

Dietary supplementation with essential fatty acids increased the accumulation and dynamics of macrophages at the inflammation. Greater and faster formation of Langhans MGCs occurred when the diet contained 100% linseed oil. Macrophage accumulation and MGC formation increased progressively until the eighth day after the implantation, corroborating the findings of Belo et al. (2005).

Table 2. Total fatty acid profile (%) of the five experimental diets.

Fatty acid	Treatments[(1)]				
	100% SO	75% SO + 25% LO	50% SO + 50% LO	25% SO + 75% LO	100% LO
C14:0 (myristic acid)	0.59	0.47	0.49	0.93	0.87
C16:0 (palmitic acid)	14.15	15.28	15.74	17.32	18.38
C16:1 (palmitoleic acid)	1.12	1.29	1.88	2.95	3.74
C17:0 (heptadecanoic acid)	0.18	0.14	0.14	0.2	0.19
C18:0 (stearic acid)	3.17	3.14	3.52	3.63	4.1
C18:1n9c (oleic acid)	24.14	25.35	27.53	29.1	31.21
C18:2n6c (linoleic acid)	47.65	37.3	21.36	12.64	3.92
C18:3n3 (linolenic acid)	5.18	14.24	26.4	28.79	33.67
C20:1n9c (eicosenoic acid)	0.34	0.28	0.3	0.36	0.34
C20:5n3 (eicosapentaenoic acid)	0.56	0.19	0.2	0.67	0.45
C22:6n3 (docosahexaenoic acid)	0.81	0.29	0.32	1.01	0.69
Not identified	2.11	2.03	2.12	2.4	2.44
Total	100	100	100	100	100

[(1)]SO, degummed soybean oil; LO, linseed oil.

Newly formed MGC have nuclei distributed randomly in their cytoplasm, which characterizes a foreign-body type of cell. Gradually, the nuclei become organized at the periphery of the cytoplasm, to form Langhans cells (Mariano & Spector, 1974). MGC formation depends on daily recruitment of monocytes from the reserve compartments of the blood. Following diapedesis and transformation into macrophages, these cells reach the inflammation focus by chemotaxis, in which IL-4 and IL-13 induce macrophage fusion (Brodbeck & Anderson, 2009) and interferon-gamma participate on Langhans giant cell formation (Anderson et al., 2008). This evolution was clearly identified in the inflammatory response of Nile tilapia. When in vitro, macrophages begin the fusion of their membranes after 24 hours in *Sparus aurata* (Couso et al., 2002), two or three days in *Cyprinus carpio* (Bayne, 1986), and after a week in *Oncorhynchus mykiss* (Secombes, 1985). Sado & Matushima (2007, 2008) observed the formation of giant cells three and seven days after the application of BCG in *Centropomus* and *Arius*, respectively. The inflammatory response reported for *P. mesopotamicus* (Petric et al., 2003a, 2003b; Belo et al., 2005) had a predominant formation of Langhans giant cells 15 days after the coverslip implantation, whereas in the present study it occurred eight days after implantation. These results show differences in the evolution of the macrophage response between different species of fish.

Comparative analysis on the cell response between the different diets clearly showed the effect of diet supplementation with linseed oil on macrophage accumulation and polykaryon formation (Table 3). Two days after implantation, a significantly greater number of macrophages and polykaryons with two nuclei was found among fish fed 75 and 100% linseed oil. On the fourth day, a greater accumulation of polykaryons was observed among fish treated with 50 and 75% linseed oil. Differences were particularly higher when compared with animals treated with 100% soybean oil.

Fish fed 100% linseed oil showed a significant increase in the formation of Langhans cells on the

Table 3. Mean values[(1)] and analysis of variance parameters of cell counts at 2, 4, 6, and 8 days after coverslip implantation in the different dietary treatments[(2)].

Treatment	Number of nuclei						
	1	2	3 to 5	6 to 10	11 to 20	>20	Langhans cell
				Day 2			
100% SO	2.16ABa	0.75ABa	0.11Aa	0.00Aa	0.00Aa	0.00Aa	0. 00Aa
75% SO + 25% LO	2.11ABa	0.84ABa	0.08Aa	0.00Aa	0.00Aa	0.00Aa	0.00Aa
50% SO + 50% LO	2.04Aa	0.53Aa	0.14Aa	0.00Aa	0.00Aa	0.00Aa	0.00Aa
25% SO + 75% LO	2.38Ba	0.97Ba	0.25Aa	0.00Aa	0.00Aa	0.00Aa	0.00Aa
100% LO	2.28ABa	0.81ABa	0.28Aa	0.00Aa	0.00Aa	0.00Aa	0.00Aa
				Day 4			
100% SO	3.01Ab	1.73Ab	1.00Ab	0.14Aa	0.00Aa	0.00Aa	0.00Aa
75% SO + 25% LO	3.09Ab	1.74Ab	1.02Ab	0.25ABb	0.04Aa	0.00Aa	0.00Aa
50% SO + 50% LO	3.08Ac	1.98Ab	1.33Bb	0.60Cb	0.18Aa	0.00Aa	0.00Aa
25% SO + 75% LO	3.06Ab	1.95Ab	1.17ABb	0.43BCb	0.04Aa	0.00Aa	0.00Aa
100% LO	3.09Ab	1.76Ab	0.98Ab	0.34ABb	0.09Aa	0.00Aa	0.00Aa
				Day 6			
100% SO	2.99Ab	1.95Ab	1.54Ac	1.18Ab	0.69Ab	0.53Ab	0.14Aa
75% SO + 25% LO	3.03Ab	2.02Ab	1.54Ac	1.07Ac	0.72Ab	0.27Ab	0.04Aa
50% SO + 50% LO	3.03Abc	2.01Ab	1.62Ac	1.16Ac	0.90Ac	0.43Ab	0.04Aa
25% SO + 75% LO	3.04Ab	1.90Ab	1.49Ac	1.10Ac	0.78Ab	0.29Ab	0.09Aa
100% LO	2.89Ab	1.95Ab	1.47Ac	1.07Ac	0.84Ab	0.52Ab	0.48Bb
				Day 8			
100% SO	2.86Ab	1.85Ab	1.43Ac	1.03Ab	0.67ABb	0.60ABb	0.45ABb
75% SO + 25% LO	2.95Ab	1.85Ab	1.41Ac	1.05Ac	0.91Bb	0.76Cc	0.38Ab
50% SO + 50% LO	2.78Ab	1.82Ab	1.38Abc	0.93Ac	0.62Ab	0.48Ab	0.62BCb
25% SO + 75% LO	2.81Ab	1.87Ab	1.35Abc	0.94Ac	0.69ABb	0.63ABc	0.69Cb
100% LO	2.91Ab	1.87Ab	1.43Ac	1.06Ac	0.88ABb	0.62ABb	0.73Cc

[(1)]Means followed by equal letters, uppercase in the columns and lowercase in the lines, do not differ by Tukey's test, at 5% probability. [(2)]SO, degummed soybean oil; LO, linseed oil.

sixth day after implantation, in comparison to the other treatments (Table 3).

After eight days, no significant difference was observed between treatments in macrophages and MCG with up to ten nuclei. However, fish fed 75 and 100% linseed oil had greater formation of Langhans cells, compared to fish fed 75 and 100% soybean oil.

According to Balfry & Higgs (2001), fatty acids in the diet can favor organism defenses, partly by helping to maintain the integrity of the lipid composition of macrophage membranes. The increased macrophage recruitment and polykaryon formation, observed in the present study, with essential fatty acid supplementation was possibly caused by the favorable action of these compounds on biological membranes, which increased chronic inflammatory response due to the foreign body. Belo et al. (2005) reported similar results with regard to vitamin E supplementation.

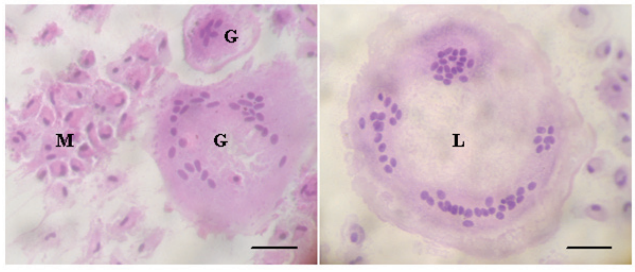

Figure 1. Photomicrograph of mononuclear macrophages (M), foreign body giant cells (G), and Langhans giant cells (L), six days after the implantation of glass coverslips in the subcutaneous tissue of the Nile tilapia (Oreochromis niloticus). Hematoxylin eosin staining. Horizontal bar = 3.0 µm.

Moreover, n-3 polyunsaturated fatty acids (Pufa) may modulate the expression of inflammatory genes downregulating some transcription factor activation (Sethi et al., 2002) and inflammatory cytokines (Zhao et al., 2005), since they may directly target the inflammatory gene expression pathways through toll-like receptor 4 (Lee & Hwang, 2006). This corroborates the hypothesis that these are active compounds on defense mechanisms among teleost fish. The increased Langhans giant cell formation on the sixth and eighth days of inflammation observed in Nile tilapia supplemented with 100% linseed shows the participation of n-3 fatty acids in the physiopathology of these processes, increasing the macrophage activity. Additionally, fatty acids that modulate immune responses and eicosanoids produced from arachidonic acid (20:4n-6) are recognized as inflammatory agents (Abeywardena & Head, 2001; Ganga et al., 2005; Calder, 2006).

Treatments did not differ in weight gain, feed consumption, apparent food conversion, and specific growth rate (Table 4). Therefore, productive performance of Nile tilapia supplemented with

Table 4. Productive performance of Nile tilapia (Oreochromis niloticus) as affected by supplementation with different proportions of degummed soybean oil (SO) and linseed oil (LO)[(1)].

Treatment	Weight gain (g)	Feed consumption (kg)	Feed conversion	Specific growth rate
		1st month		
100% SO	46.61±6.38Aa	0.66±0.02Aab	1.50±0.20Ab	1.27±0.23Aa
75% SO + 25% LO	46.16±4.22Aa	0.59±0.09Aab	1.27±0.11Ab	1.25±0.11Aa
50% SO + 50% LO	45.32±3.02Aa	0.60±0.04Aa	1.31±0.21Ab	1.28±0.14Aa
25% SO + 75% LO	45.09±6.33Aa	0.70±0.01Aab	1.63±0.20Ab	1.13±0.24Aa
100% LO	40.88±7.01Aa	0.59±0.10Aa	1.46±0.21Ab	1.00±0.18Aa
		2nd month		
100% SO	40.69±2.30Aab	0.81±0.03Aa	2.00±0.04Aa	0.84±0.03Ab
75% SO + 25% LO	38.71±11.55Aa	0.80±0.15Aa	2.09±0.17Aa	0.82±0.17Ab
50% SO + 50% LO	39.37±3.55Ab	0.85±0.10Aa	2.14±0.11Aa	0.84±0.05Ab
25% SO + 75% LO	48.05±11.00Aa	0.88±0.11Aa	2.10±0.42Aa	0.89±0.18Ab
100% LO	37.97±5.12Aa	0.86±0.11Aa	2.27±0.06Aa	0.76±0.08Ab
		3rd month		
100% SO	26.71±2.77Ab	0.50±0.02Ab	1.92±0.20Aab	0.91±0.08Ab
75% SO + 25% LO	27.83±3.28Aa	0.45±0.03Ab	1.67±0.19Ab	0.84±0.06Ab
50% SO + 50% LO	28.34±3.80Ac	0.52±0.05Ab	1.88±0.14Aab	1.01±0.08Aab
25% SO + 75% LO	25.83±2.72Ab	0.56±0.09Ab	2.10±0.16Aa	0.95±0.14Ab
100% LO	24.48±5.61Ab	0.41±0.03Ab	2.01±0.47Aab	0.85±0.11Ab

[(1)]Means followed by equal letters, uppercase in the columns and lowercase in the lines, do not differ by Tukey's test, at 5% probability.

different essential fatty acids was similar. This result was also obtained in different situations, with different species (Bell et al., 2003; Mourent et al., 2005; Vargas et al., 2008).

Conclusions

1. Nile tilapia (*Oreochromis niloticus*) diets supplemented with high levels of linseed oil increase macrophage activity, with greater macrophage accumulation and faster formation of MGC and Langhans giant cells at the beginning of the inflammation.

2. Linolenic acid (C18:3, n-3) supplementation benefits these nonspecific defense mechanisms.

3. Productive performance of Nile tilapia fed n-3 or n-6 fatty acids does not differ.

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